APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1982, p. 334–338
0099-2240/82/080334-05$02.00/0

Vol. 44, No. 2

Metabiotic Effect of *Bacillus licheniformis* on *Clostridium botulinum*: Implications for Home-Canned Tomatoes

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Received 24 February 1982/Accepted 20 April 1982

The metabiotic effect of *Bacillus licheniformis* on *Clostridium botulinum* was examined. *B. licheniformis* elevated the pH of a model system with an initial pH of 4.4 so that *C. botulinum* grew and produced toxin. Toxin production was observed when spores from both species were coinoculated at levels as low as 10 spores per ml. When pint jars of tomatoes were used, canner size contributed to a 10,000-fold difference in the lethality of a boiling water bath process on *B. licheniformis* spores. Botulinal toxin was not detected in pH-elevated jars of tomatoes containing *C. botulinum* spores.

The adequacy of home canning practices used to preserve tomatoes has been questioned for many years. Because a small fraction of tomatoes have a pH ≥4.8 and might support the growth of *Clostridium botulinum*, acidification as an adjunct to the boiling water bath process has been suggested (20, 21). The effect of acidification on the quality of canned tomatoes has been reviewed (18).

Metabiosis, the ability of one species to change otherwise unfavorable conditions and thereby allow a second species to grow, is one means by which *C. botulinum* can grow in acid foods (15). Metabiosis has been observed with postprocess contamination of tomatoes by molds which elevate the pH and allow subsequent toxin production by *C. botulinum* (10, 16).

An earlier paper from this laboratory (12) reported that *Bacillus licheniformis* isolates, which are frequently found in tomatoes (5), have spores that might survive the U.S. Department of Agriculture raw-pack process for tomatoes, raise the pH, and allow toxin production by *C. botulinum*. The purpose of this paper is to report that metabiosis by *B. licheniformis* can permit toxin production by *C. botulinum*. The ability of *B. licheniformis* spores to survive the boiling water bath process for tomatoes currently recommended by the U.S. Department of Agriculture and to cause pH elevation is also reported.

MATERIALS AND METHODS

Organisms and culture conditions. Spores of *C. botulinum* strains 62A, ATCC 25763, B-aphis, and 53B were prepared as previously described (11), diluted in distilled water to ~10^6/ml, and stored at ambient temperature under anaerobic conditions.

The preparation of spores of *B. licheniformis* strains 075-T-09, 011-T-11, 110-T-05, and 015-T-03 was as described by Montville and Sapers (12). These strains originally were isolated from home-canned tomatoes by Fields et al. (5).

Heat-processed tomatoes were tested for viable *B. licheniformis* by the three-tube most-probable-number method by using tryptic soy broth (Difco Laboratories) incubated aerobically at 35°C for 72 h. Pellicle formation was used as a presumptive indicator for *B. licheniformis*. Isolates from all positive most-probable-number tubes were subsequently confirmed as *B. licheniformis* by biochemical tests (8).

Postprocess counts of *C. botulinum* were determined directly from spread plates on botulinum assay medium (BAM) (9, 11) incubated anaerobically at 35°C for 72 h. Anaerobic conditions were obtained in a chamber (Coy Laboratory Products) similar to that of Aranki et al. (3) charged with a mixture of 90% N_2, 5% CO_2, and 5% H_2.

Model system experiments. Double-strength BAM containing 0.018 g of phenol red per liter and double-strength tomato juice broth (TJA) (Difco) were adjusted to pH 4.4 with 0.1 N HCl. Each medium was dispensed into culture tubes (20 by 150 mm; 5 ml of medium per tube) fitted with permeable membrane caps (Kimble), and the tubes were then sterilized. Immediately before inoculation, 5 ml of sterile agar (3% in distilled water) was added to each tube. Equal numbers of heat-shocked (80°C for 10 min) *C. botulinum* 62A and *B. licheniformis* 075-T-09 spores were added to the tubes of molten agar in the anaerobic chamber. Triplicate tubes were coinoculated with 10^2, 10^4, or 10^6 spores of each organism. Controls were triplicate tubes inoculated with 10^6 *B. licheniformis* or *C. botulinum* spores or left uninoculated. The tubes of solidified medium were incubated aerobically at 30°C. Pre- and postincubation pH measurements were made with a digital pH meter with slope control (Sargent Welch) and a flat membrane combination electrode (Fisher Scientific Co.) standardized against buffers at pH 4.00 and 7.00.

Inoculated pack experiments. Pint jars of Roma VF tomatoes (pH 4.4) were inoculated along the vertical axis with *C. botulinum* and *B. licheniformis* spores in combination or singly. Equal numbers of spores from the four *C. botulinum* strains were used to give a total

TABLE 1. pH and toxicity of BAM inoculated with *C. botulinum* 62A and *B. licheniformis* 075-T-09 spores after 8 days of aerobic incubation at 30°C

Inoculum level (spores per ml)		pH		No. of triplicate tubes with botulinal toxin
B. licheniformis	*C. botulinum*	On surface	In zone of anaerobic growth	
0	10^5	4.4	ND[a]	0
10^5	0	7.6	ND	0
10	10	8.2	6.2	3
10^3	10^3	7.9	5.8	3
10^5	10^5	8.5	6.5	3

[a] ND, Not done because no anaerobic growth was observed.

of 10^6 spores per jar. Spores from the four *B. licheniformis* strains also were mixed to give a total of 10^6 spores per jar. These jars and uninoculated controls were processed by the U.S. Department of Agriculture recommended procedure (35 min in a boiling water bath) for raw-pack tomatoes (22). To simulate leakers, the seals on half of the processed jars were broken under a laminar flow hood. All of the jars were incubated at 30°C.

Heat penetration into pint jars of tomatoes in a boiling water bath was determined with a telethermometer (Yellow Springs Instrument, model 42SC) whose thermocouple was placed at the geometric center of the jar, which is the cold spot in conduction-heated foods. Experimental agreement between the calculated and actual process lethalities (see below) justified the assumption of conduction heating. The heat penetration data were used with previously determined decimal reduction time (D) and z values (12) to calculate the lethality of the boiling water bath process for *B. licheniformis* spores.

Detection of botulinal toxin. Samples from the agar tubes were prepared after the top alkaline portion which contained the *B. licheniformis* was discarded. The agar containing the zone of anaerobic growth was then macerated with 10 ml of gel phosphate buffer (4). The supernatant obtained by centrifugation at 8,740 × *g* for 90 s in a Microfuge B (Beckman Instruments, Inc.) was used for the toxin assay. The entire contents of each tomato jar were blended for 60 s, and a portion was centrifuged as described above to prepare the samples for toxicity tests.

Each sample was tested for botulinal toxin by injecting each of two 15- to 20-g Swiss white mice intraperitoneally with 0.4 ml of the putative toxin. The mice were observed for symptomatic botulinic death for 72 h. Control samples that had been boiled for 10 min did not kill mice. All deaths attributed to botulinal toxin occurred within 24 h.

RESULTS

Model systems. All BAM and TJA tubes inoculated with *B. licheniformis* spores exhibited confluent surface growth within 4 days of incubation. A pH gradient within the tubes was demonstrated by the spectrum of phenol red reactions which ranged from red (alkaline) at the top to yellow (acidic) at the bottom of the tubes. After 8 days of incubation, zones of anaerobic growth were observed in the coinoculated tubes of BAM. These zones did not visibly disrupt the continuity of the color spectra (pH gradients). Although *B. licheniformis* is classified as a facultative anaerobe (7), no anaerobic growth was observed in tubes inoculated with only *B. licheniformis*. Tubes inoculated with only *C. botulinum* spores were negative for growth.

The metabiotic effect was easily visualized in the BAM model system (Fig. 1). *B. licheniformis* grew at the surface, and discrete colonies and a zone of anaerobic growth were seen in tubes that were also inoculated with 10^4 or 10^6 *C. botulinum* spores. Quantitative pH data (Table 1) demonstrated a pH gradient in tubes inoculated with *B. licheniformis*.

After 30 days of incubation, the zone of anaerobic growth had increased in size, the tubes had a distinctly clostridial odor, and the pH at the surface of the tubes had dropped from the day 8 value of >7.0 to a value of 5.2 to 6.4. Botulinal toxin was detected in all of the coinoculated BAM tubes (Table 1). This confirmed that the anaerobic growth was *C. botulinum*.

Only one of the coinoculated TJA tubes (Fig. 1, tube 5) developed a zone of anaerobic growth. This zone did not become larger during prolonged incubation. No toxin was detected in this sample.

Inoculated packs. No viable *B. licheniformis* spores could be isolated from 24 jars of tomatoes processed in a large canner during three separate trials. This nine-place, 15-in. (ca. 38-cm)-diameter canner held 16 liters of water when filled to 1.5 in. (ca. 3.8 cm) above the top of an eight-jar load.

B. licheniformis spores survived the processing of tomatoes in a smaller canner but grew out and elevated the pH only when the seal was broken to simulate leakers. The seven-place, 12-in. (ca. 30.5-cm)-diameter canner held 11 liters of water when filled to cover a six-jar load. The postprocess most probable number for inoculated jars was 4.0×10^2 *B. licheniformis* spores per jar, a 3.3-log reduction from the initial number.

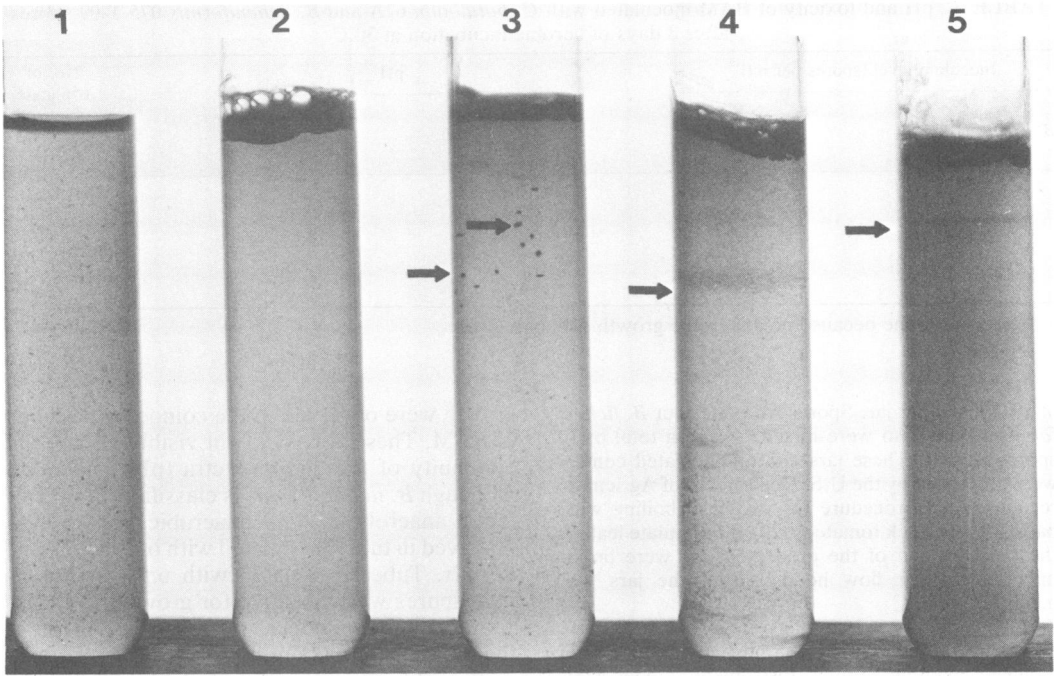

FIG. 1. Growth patterns in tubes of BAM (tubes 1 through 4) or TJA (tube 5) inoculated with nothing (tube 1), 10^6 *B. licheniformis* spores (tube 2), 10^4 *B. licheniformis* spores and 10^4 *C. botulinum* spores (tube 3), or 10^6 *B. licheniformis* spores and 10^6 *C. botulinum* spores (tubes 4 and 5) and incubated aerobically at 30°C for 8 days. Arrows point to zones or colonies of *C. botulinum*.

The postprocess *C. botulinum* level was 1.2×10^5 per jar, slightly more than a 1-log reduction. Jars of tomatoes in which *B. licheniformis* grew out developed a pH gradient with values of 7.35 to 7.55 at the surface, 4.76 to 6.20 in the middle of the jar, and 4.50 to 4.68 at the bottom. The pH of the blended contents was 4.78 to 5.90. No growth or pH elevation was observed in jars with sound seals.

One of twelve coinoculated leakers with elevated pH had a clostridial odor after 30 days of incubation, but toxin was not detected in any of the jars. Trypsinized samples also gave negative assays for botulinal toxin.

Although the time required for the water to resume boiling after the jars were placed in either canner differed, processing in both canners met the recommended 35 min in a boiling water bath. Since the different results obtained with jars processed in the large and small canners could have been due to different come-up times (the time required for boiling to resume) heat penetration profiles were determined. The two sets of jars had the same rate of heat penetration (Fig. 2), but jars in the large canner received a longer total process (time required for boiling to resume plus boiling time) because the 35-min process starts when the water resumes boiling. The added time is at a temperature at which the lethal rate is large and contributes significantly to the process lethality. The final internal temperature of jars in the large canner was 7°C higher than that of jars in the small canner. This added to the lethality contributed during the cooling period. Since the lethal rate at 63°C was 0.001 log per min, the time above this temperature was used to calculate the total process lethality for jars in each canner. The calculated lethalities for *B. licheniformis* spores were 2.7 decimal reductions for the small canner and 7.4 decimal reductions for the larger canner. These values compare favorably with the most-probable-number survival data and justify the assumption of conduction heating which was used to determine the thermocouple placement.

DISCUSSION

The metabiotic effect of *B. licheniformis* on *C. botulinum* observed in the BAM model system is in contrast to the antagonistic effect in brain heart infusion broth, where bacitracin produced by *B. licheniformis* inhibits botulinal growth and toxin production (23). These reports are not necessarily contradictory. Physical parameters, such as whether *B. licheniformis* and *C. botulinum* share the same microenvironment (as in a broth) or are separated by a diffusable barrier (as in an agar), may be important determinants in

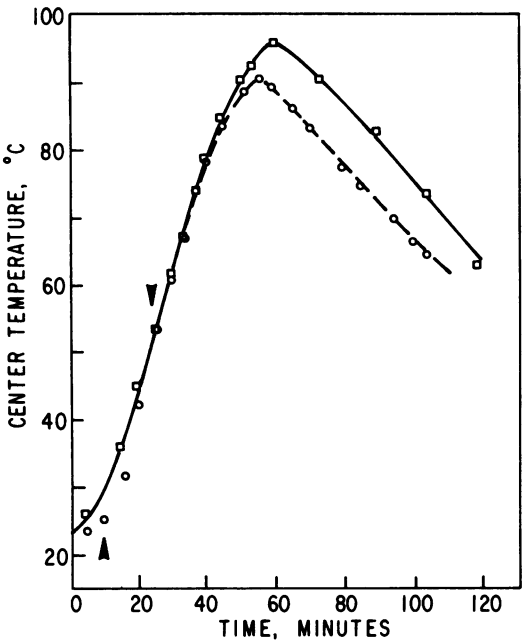

FIG. 2. Heat penetration curves for pint jars of tomatoes processed in a large (□) or small (○) canner (see text.) ▲ and ▼, resumption of boiling in the small and large canner, respectively.

the interaction. Bacitracin is unstable at 35 to 37°C (2). It may have been inactivated before it could diffuse through the agar to the zone of botulinal growth. It is also possible that BAM is a poor medium for bacitracin production.

The conditions necessary for *B. licheniformis* to create a potential botulism problem in canned tomatoes can exist in practical situations. *Bacillus* spp. have been isolated from 40% of home-canned tomatoes (5). They survived the recommended heat processing when a smaller canner was used. Since the vacuum of home-canned tomatoes ranges from 0.0 to 27.1 in. (ca. 69 cm) of mercury (19) and since 1.1% of the jars tested have a poor seal or low vacuum (13), the aerobic conditions needed for growth of the organism are satisfied in some home-canned preparations.

B. licheniformis spores survived the processing of tomatoes in the small canner. This is of some concern. *B. licheniformis* spores, with a D_{95} of 5.1 min in tomatoes (12), are not very heat resistant compared with *Bacillus coagulans* spores, which have a D_{95} of 13.7 min (1). This indicates that the boiling water bath process currently recommended is ineffective against heat-resistant spores if small water baths with short come-up times are used. Spoilage by heat-resistant species such as *B. coagulans* would lower the product pH (6) and would not pose a botulinal hazard.

Although toxin was not detected in coinoculated tomatoes, the observation of metabiosis is still a matter of concern. The detection of a clostridial odor in one of the jars suggests that *C. botulinum* did grow in a canned tomato system. Tomatoes with elevated pH are a suitable medium for toxigenesis by *C. botulinum* (10, 16).

There are several possible reasons why toxin was not detected. There may be only a short period when the pH is favorable for growth of *C. botulinum*. This hypothesis is supported by the observation that the relatively small zone of anaerobic growth in the TJA tube did not increase in size upon prolonged incubation. The zones in the BAM model system did increase. It is also possible that the toxin, once formed, may be inactivated by increasingly alkaline conditions. Although tomatoes are highly buffered (17), once the buffering capacity is exceeded, the pH can rise rapidly.

The metabiotic interaction in canned tomatoes may be a low-probability event which is difficult to detect in laboratory trials. Given the large number of jars of tomatoes which are home processed, such low-probability events can be expected to occur eventually. The metabiotic effect may be more probable in other marginally acidic foods which have different buffering capacities. pH elevation of acidified onions by *B. licheniformis* has been reported (14). The observation that canner size is an important determinant of process lethality was unexpected. The lethality of the process to *B. licheniformis* can be increased 10,000-fold by the 15-min increase in the total process time which is dictated by how long it takes the water in the canner to reboil (Fig. 2). The generation and use of different recommendations for different canner sizes would be cumbersome and undesirable. A closer examination of the heat penetration profiles indicates that this may not be necessary. The heat penetration profiles for the jars in each canner are the same, regardless of how long it takes for boiling to resume. If the process recommendation had been based on total time of immersion rather than on time from resumption of boiling, jars in these experiments would have received the same lethality regardless of canner size.

In their review of *C. botulinum* in acid foods, Odlaug and Pflug (15) state that "an acid food is safe from *C. botulinum* if the heat process kills all organisms capable of growing at pH $\leq$4.6 and there is no post-process contamination." The current procedure recommended by the U.S. Department of Agriculture does not assure that all organisms capable of growing at pH $\leq$4.6 are killed. The data suggest that that addition of 10 min to the process time for the raw-pack canning of pint jars of tomatoes may be justified. This would increase the lethality of the process 1,000-

fold with respect to *B. licheniformis*. A longer process would provide an added safeguard against the possibility of metabiosis resulting in growth and toxigenesis by *C. botulinum* and would result in reduced losses due to spoilage.

In summary *B. licheniformis* could elevate the pH and deplete oxygen in an aerobically incubated acidic model system, thereby allowing growth and toxin production by *C. botulinum*. Canner size had a large effect on the lethality of a boiling water bath process to *B. licheniformis*. Even if there is no botulinic threat from tomatoes processed by the current recommendations, consideration should be given to the adoption of a longer process which should result in reduced losses due to spoilage. The exact specifications of a longer process should be the subject of further study.

ACKNOWLEDGMENTS

I thank G. M. Sapers for encouragement and helpful discussions during this study, L. Conway, G. Maher, and A. DiVito for valuable technical assistance, and D. D. Bills, J. L. Smith, and C. N. Huhtanen for helpful comments on the manuscript.

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